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PRODUCTION AND CHARACTERIZATION OF CHICKEN IMMUNOGLOBULIN Y (IGY) AGAINST *ESCHERICHIA COLI* O157: H7, *SALMONELLA TYPHIMURIUM*, *GALLIBACTERIUM ANATIS* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

This study aimed to produce hyper-immune eggs against several economically important bacteria in birds. These bacteria are *Escherichia coli* O157: H7, *Salmonella typhimurium*, *Gallibacterium anatis* and *Staphylococcus aureus*, which were isolated from diseased broiler chickens. The purity of isolated IgY was assessed by SDS-PAGE and the specificity to the bacteria was evaluated by growth inhibition assay. The results indicated that serum antibodies against those bacteria reduced the bacterial growth from the first week till reaching a peak of inhibition at the 4th week and the inhibition continued up to the 8th week post immunization.

The egg yolk antibody bacterial growth inhibition started from the second week and reached peak at the 5th week and the inhibition continued up to the 8th week post immunization. Thus, using egg yolk antibodies for management of *E. coli* O157: H7, *S. typhimurium*, *G. anatis* and *S. aureus* infections in broilers can be of good field application values.

KEYWORDS: IgY, *E. coli*, *Salmonella*, *S. aureus*, *G. anatis.*

INTRODUCTION

There are many bacteria causing serious problems to the poultry industry resulting in high economic losses such as *Escherichia coli* O157: H7 **(*E. coli*)**, *Staphylococcus aureus* **(*S. aureus*),** *Gallibacterium anatis* **(*G. anatis*),** and *Salmonella typhimurium* **(*S. typhimurium*).**

E. coli O157:H7 is a gram-negative bacterium that has become an important food and waterborne pathogen **(Lim et al., 2010).** It causes diarrhea, hemorrhagic colitis in animals, and diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome **(HUS)** in humans **(Whittam et al., 1988; Dipineto et al., 2006)**. It showed resistance to tetracycline, sulfonamides, erythromycin, streptomycin, cephalothin, gentamicin and ampicillin. Resistance to tetracycline was the most common finding, followed by resistance to sulfamethoxazole, chloramphenicol and trimethoprim **(Momtaz et al., 2012).**

S. typhimurium lives in the intestinal tracts of warm and cold-blooded animals **(Schlundt, 2002)**. It is the major cause of food-borne disease throughout the world **(Humphrey, 2002)**. *Salmonella* infected poultry and poultry products represent a source of pathogens for humans through transportation and consumption of undercooked poultry meat, causing severe illness and even death **(Bailey and Cosby 2003; Kimura et al., 2004; Wang et al., 2008).**

S. typhimurium infection in chicken can occur at any age, but the infection mainly causes systemic disease with high mortality in day-old chicks as they are more susceptible to the infection **(Shao et al., 2013)**. However, *S. typhimurium* infection in older birds usually causes gut inflammation, intestinal barrier damage, poor growth rate, and reduced egg production **(Dar et al., 2017)**. *S. typhimurium* showed resistance to doxycycline, ampicillin, gentamycin, colistin, vancomycin and neomycin **(Yu et al., 2008).** In human, the infection is characterized by a variety of clinical manifestations including encephalopathy, peritonitis, perforation and

hemorrhage **(Baggesen et al., 2002; Aktas et al., 2007).** *Salmonella* is major food-borne pathogen in most countries especially in developing ones **(Soultose et al. 2003; Carraminanaet al., 2004).**

G. anatis lives in the upper respiratory tract and the lower genital tract of healthy chickens **(Bojesen et al., 2003; Christensen et al., 2003)**. It has been reported to be associated with bacteremia, oophoritis, follicle degeneration, salpingitis, peritonitis, hepatitis, enteritis, and respiratory tract diseases in chickens **(Proctor et al., 2006)**. It causes loss in production with heavy mortality in broiler chicken and drop in egg production in layers with increased mortality **(Bojesen et al., 2008)**. Also, it infect turkeys, geese, ducks, pheasants, partridges, budgerigars, peacock, cage birds, wild birds, cattle and pig **(Rzewuska et al., 2007)**. *G. anatis* are highly sensitive to cefotaxime, gentamycin, amoxicillin and ampicillin, moderately sensitive to doxycycline, norfloxacin, florfenicol and ciprofloxacin, and resistant to erythromycin, cephradin, oxytetracyclin, sulphatrimethoprim, streptomycin, lincomycin, spectinomycin as reported by Bojesen et al. **(2011)** and Elbestawy et al. **(2018)**.

S. aureus is a gram-positive bacterium arranged in grape-like clusters **(Kloos et al., 1994)**. It is one of the most prevalent pathogens in both animals and humans **(Casey et al., 2007)**. In poultry, *S. aureus* is associated with many clinical syndromes including tenosynovitis, omphalitis, femoral head necrosis, infected hock and bumble foot **(Suleiman et al., 2013; Abd El Tawab et al., 2017)**. In animals, *S. aureus* elicits mastitis **(Haenni et al., 2014)**, bacteremia, pneumonia, septic arthritis, omphalophlebitis and osteomyelitis **(Weese et al., 2005)**. There is growing resistance of *S. aureus* to many antimicrobial agents such as β- lactams, Macrolides, Aminoglycosides, tetracyclines and many others **(Bakheet et al., 2018)** leading to complications in the treatment of its infections as well as increasing the cost of treatments. In humans, *S. aureus* is a major pathogen responsible for both nosocomial and community-acquired infections **(Francois et al., 2005)**, including skin and wound infections, toxic shock syndrome, arthritis, endocarditis, osteomyelitis, and food poisoning **(Gao and Stewart, 2004; Von Eiff et al., 2001)**.

The benefits of IgY technology and its universal application in both research and medicine is expected to expand at large-scale. These antibodies could help address the emergence of drug-resistant microorganisms worldwide and the consequent reduction in the use of antibiotics, with the wide increase of antimicrobial resistance **(Guimaraes et al., 2009)**.

Hens' eggs have long been identified as an excellent source of human nutrients, as well as a valuable source of antibodies, the most abundant of which is immunoglobulin Y **(Yegani and Korver, 2010)**. Recently, this characteristic has attracted an increasing interest by scientists. The concentration of IgY in the egg is closely related to that in the maternal serum **(Hamal et al., 2006)**. Thus, by means of immunizing laying hens with a certain target antigen, their immune system as well as the composition of the antibodies pool can be controlled, initially in the serum, then in the eggs **(Xu et al., 2011)**. The specific antibodies obtained can then be used to immunize other individuals or feed additive. They offer as well, a solution for the incapability to treat or prevent certain diseases with traditional vaccines in certain production sectors, as in industrial broiler chickens that their life cycle is restricted to about 42 days **(Namata et al., 2009)**.

The increasing interest in IgY technology comes from its many advantages compared to its mammalian equivalent, IgG. The first advantage of getting IgY through laying hens as an alternate to mammals, is better animal welfare **(Schade et al., 1996)**. Because, unlike the mammalian models, it does not involve bleeding of the antibody producing animals. The long-lasting titers got from laying hens also reduce the need for repeated booster injections **(Schade et al., 2005)**. Another advantage is that laying hens can produce Ig in higher quantities e.g., 5-6 times higher than a rabbit **(Narat, 2003)**, which considerably lowers the number of required animals to get the antibodies. Further, the IgY extraction processes are used so far both efficient and inexpensive (De Meulenaer et al., 2001).

The hyper-immune yolk can as well be used simply as it is. The use of antibodies obtained from the egg is thus less labor-intensive and more cost-effective than traditional production of Ig from mammals. The use of IgY is environmentally friendly and does not cause unwanted side effects, disease resistance or toxic remains **(Coleman, 1999)**.

The chicken egg yolk antibodies **(IgY)** have been successfully used for scientific, diagnostic, prophylactic and therapeutic purposes. Here we studied simple and efficient production of IgY antibodies against *E. coli* O157: H7, *S. typhimurium*, *S. aureus* and *G. anatis* as well as studying few immunogenic characters of the extracted IgY that can be possibly used in therapeutic and protection approaches in order to overcome the wide antimicrobial resistance shared by the used bacteria. Controlling antimicrobial resistance can help greatly in reducing the zoonotic threat to human by such pathogens.

MATERIALS AND METHODS

Bacterial isolates

E. coli O157:H7 was isolated from chicken and cultured on MacConkey agar, incubated for 24 hours at 37^{o}C and confirmed by biochemical analysis **(Alnahass et al., 2016)**.

S. typhimurium was kindly provided by the Deprtment of Microbiology, Faculty of Veterinary Medicine,

Alexandria University. It was cultured then confirmed by biochemical analysis according to Abd Allah et al., **(2018)**.

Gallibacterium anatis biovar *haemolytica* strain B14 **(accession No: KJ026147)** was cultured on blood agar, incubated for 24 hours at 37^{o}C under microaerophilic condition and confirmed by biochemical analysis **(Elbestawy et al., 2018)**.

S. aureus was isolated from broiler chickens suffering from swollen hock joints and pododermatitis. The samples were collected from the swollen joints then inoculated into brain heart infusion broth and incubated at 37^{o}C for 18–24 hours. A loopful from each sample was plated onto Baired-parker agar medium and Mannitol salt agar medium. The inoculated plates were incubated at 37^{o}C for 24–48 hours under aerobic conditions. Suspected colonies were picked up for purification then sub-cultured on blood agar and nutrient agar media and subjected for identification **(cultural, microscopical and biochemical) (Quinn et al., 2002, Boerlin et al., 2003, El-Masry et al., 2016, Ali et al., 2017 and Mohamed et al., 2019)**. Purified bacterial colonies from those 4 types of bacteria were cultured in nutrient broth, incubated for 24 hours at 37^{o}C. Bacteria were harvested by centrifugation at 5,000 g for 15 min, washed three times, suspended in sterile saline, subjected to repeated freezing and thawing **(Sunwoo et al., 2010)**.

Experimental birds

A total of 16 commercial layer balady chickens aged 32 weeks old **(initially weighing 1500 g)** were purchased from a local farm and housed in animal facility joined with Microbiology Department, Faculty of Veterinary medicine, Damanhour university. They were left for 4 weeks for acclimatization and kept under a lighting regimen for 16 hours in clean disinfected rooms and supplied with standard layer 1 ration **(containing 18% protein and 2850 Kcal)** for layers at a rate of 110 g/bird /day and water *ad libitum* **(Nahla et al., 2018 and Hezema et al., 2020).**

Bird immunization

Hens were divided into four groups each containing 4 hens and each group was immunized with one type of bacteria. Hens were intramuscularly injected in the breast muscle with bacteria emulsified with equal volume of Freund's complete adjuvant **(Sigma-Aldrich)** as the first dose then subsequently followed by two booster doses with incomplete Freund's adjuvant given 2 and 4 weeks after the initial dose **(Wooley and Landon, 1995; Zhen et al., 2008 and Nahla et al. 2018)**. All the vaccinal preparations were prepared according to Moncada et al. **(1993)**.

Collection of samples

Eggs and serum one day before immunization of hens were collected to be used as negative controls. Regular blood sampling was done every week after final injection from the wing vein. Blood was collected then left to coagulate and then centrifuged at 3000 r.p.m for 30 minutes for separation of serum. The collected sera were stored at -20^{o}C till used for further analysis. Eggs were collected daily throughout the entire period of the experiment **(8 weeks)**. The egg was cracked, opened under hygienic condition and egg white was discarded, and the yolk was collected, washed with PBS and stored at -20^{o}C until processed for purification **(Wooley and Landon, 1995, Zhen et al., 2008, Abo-Ghanema et al., 2016, Nahla et al., 2018 and Hezema et al., 2020*)***.

Extraction and purification of Ig Y

IgY antibody was extracted and purified from egg yolks according to Akita and Nakai, **(1993)** and Nahla et al., **(2018).** Briefly, 5 g of egg yolk was 6 folds diluted with 10 mM phosphate buffer **(PH 5- 5.2)** and homogenized thoroughly using vortex. The sample mixture was centrifuged at 12,000 x g for 30 minutes at 4°C to remove the lipid-rich precipitate. The supernatant, consisting of the lipid-free fraction, was collected and precipitated with 40% ammonium sulfate **(w/v)**. After centrifugation **(12,000 x g for 30 minutes at 4°C),** the pellet containing the IgY enriched fraction was dissolved in 2ml PBS. To eliminate the residual salt, the purified IgY was dialyzed against PBS for 24 hours. The final IgY was stored at -20°C.

Assessing the purity of the isolated IgY by SDS-PAGE

The purified IgY fraction and serum IgY were subjected to SDS-PAGE analysis according to the method adopted by Laemmli, **(1970),** Muhammad et al., **(2018).**

Bacterial growth inhibition

This assay was performed according to Thibodeau et al., **(2017)** with some modifications. Briefly, the bacterial culture was mixed with 2 ml of nutrient broth and incubated at 37^{o}C for 24 hr. Then 50 µL of the bacterial suspension was mixed with 50 µL of purified IgY and incubated at 37℃ for 1 hr, the bacterial suspension was spread onto nutrient agar plates and incubated for 24 h at 37℃. Plates were visually monitored for bacterial growth.

Agar gel immunodiffusion test for *Gallibacterium anatis* biovar *haemolytica* It was carried out according by Ouchterlony & Nilsson, **(1986)** to detect the specific antibodies against the bacterial pathogens in hens' serum and purified egg yolk. Agar plates were prepared with 0.7% agar **(Agarose 1, biotechnology grade, Amresco, Solon, OH, USA)** dissolved in borate buffer solution **(0.2% NaOH, 0.9% H3BO3, pH 8.6)** containing 7.0% NaCl. Forty microliters of the bacterial prepared antigen were pipetted into the center well and the tested serum was placed in the surrounding wells. Plates were read at 24 h and 48 h. Test was considered positive if a line of precipitation was fully formed between the test well and the antigen well.

RESULTS

Assessment of IgY purity

The purified IgY fraction from yolk and serum against *E. coli* O157:H7, *S. typhimurium*, *G. anatis* and *S. aureus* were subjected to SDS-PAGE analysis under reducing conditions. The antibody showed two major bands with a molecular weight ranging from 26 to 27 KDa corresponding to the light chain, and from 63 to 69 KDa corresponding to the heavy chain (Figure 1).

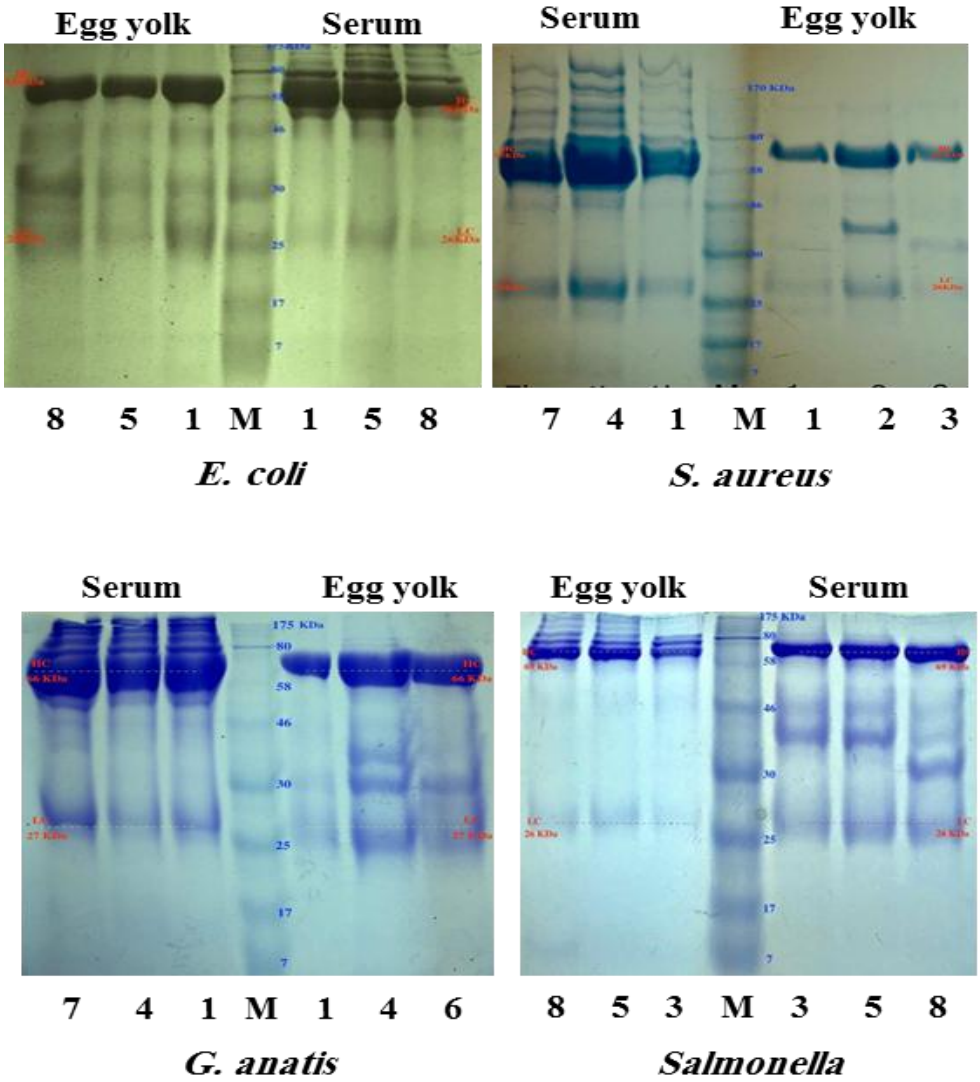

Figure (1): SDS-PAGE analysis of purified egg yolk and serum IgY against *E. coli* O157:H7, *S. typhimurium*, *G. anatis* and *S. aureus*, showing two major bands with molecular weight of 26~ 27 KDa and 63~ 69 KDa corresponding to light and heavy chains, respectively. M; molecular weight marker. Different numbers below the photos indicate the weeks of the tested egg yolk and serum samples.

Growth inhibition assay

The inhibition of bacterial growth by serum and egg yolk IgY started from the second week after immunization, and increased gradually till reaching peak at the 5th week, and continued up to the 8th week post immunization against *E. coli* O157:H7, *S. typhimurium*, *G. anatis* and *S. aureus* as shown in **Figure 2**.

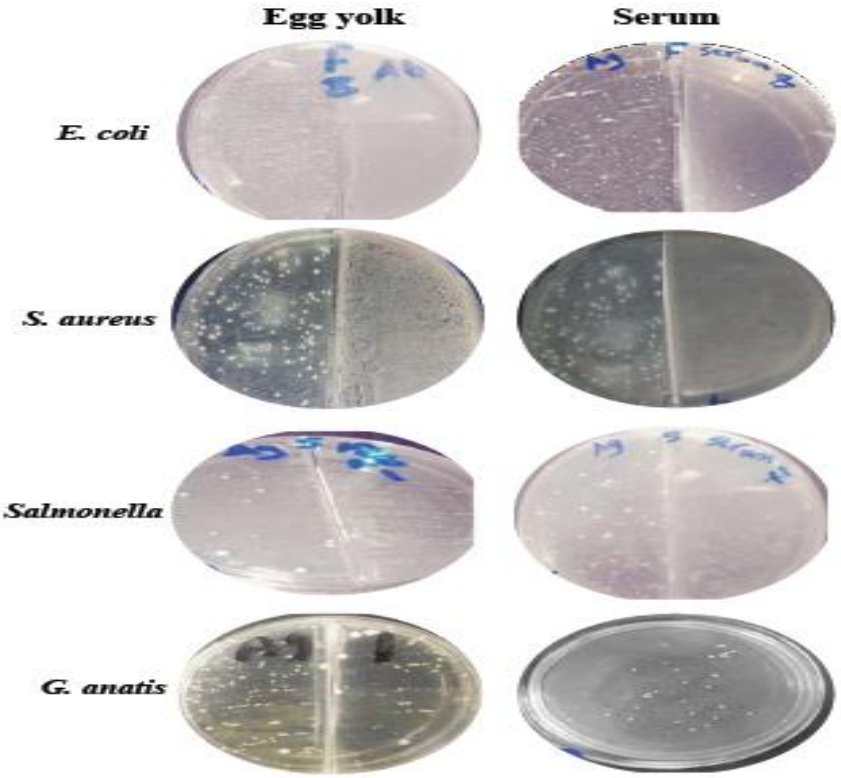

Figure (2): Bacterial growth inhibition by egg yolk and serum IgY produced against *E. coli* O157:H7, *S, typhimurium, G. anatis* and *S. aureus*. Bacteria cultured without antibodies is shown on the left side of the plate, bacteria cultured with antibodies is shown on the right side of the plate.

Agar gel immunodiffusion test

Immunoprecipitation characteristics of antibodies in both serum and yolk sample produced against *G. anatis* were tested. For serum samples, As shown in **Figure 3**, precipitation lines appeared first at the third week and continued up to the seven-week post-immunization.

Disappearance of precipitation lines occurred thereafter. In contrast, no precipitation lines could be detected between the *G. anatis* antigen and purified yolk IgY at different weeks post immunization.

Figure (3): Agar gel immunodiffusion test against *G. anatis* serum IgY. Precipitation lines appeared as white lines between the antigen and the antibody wells. A; *G. anatis* antigen, numbers 2-7; weeks of collection of serum.

DISCUSSION

Several studies have been done to explore the usefulness of avian immunoglobulin Y (IgY) in food, microbial, and residual analyses **(De Meulenaer et al., 2002; Pauly et al., 2009; Sunwoo et al., 2006)**, immunodiagnostics, passive immunization, and therapeutic functions **(Hirai et al., 2010; Lee, 2008).** Its application as an immunotherapeutic agent for oral passive immunization against enteric pathogens was reported. Further, they do not activate mammalian, nor do they interact with mammalian Fc receptors, which could mediate gastrointestinal inflammatory reactions. IgY has also been shown to be advantageous in many techniques and in the purification of immune affinity in several cases replacing IgG due to its distinctness from IgG. Moreover, the continuous production of antibodies in chicken is advantageous **(Dias da Silva and Tambourgi, 2010).**

Previous results showed that the intramuscular route results in higher antibody levels with high specificity **(Li et al., 2019)**. On the other hand, **Schwarzkopf et al., (2000)** showed that the S/C antigen injection provoked a higher antibody titer than the I/M route of injection. In the intramuscular route, the immune response seems to work earlier than in the subcutaneous route, but eventually the subcutaneous route reaches the same level, and even higher **(Salomo, 2015).**

Laying hens are highly cost effective as producers of

antibodies compared to mammals **(Larsson et al., 1993).** Average volume of egg yolk (15 mL) contains 50-100 mg of IgY, of which 2%–10% can be of specific antibodies, this is a much higher amount of immunoglobulin that could be obtained by bleeding the animal **(Sudjarwo, et al., 2012; Ferreira et al., 2012).** The IgY has been applied successfully in diagnostics, prophylactic, therapeutic purposes, immunochemical reagents and in food formulation or supplements due to it is stability under food processing conditions **(Raj et al., 2004; Schade and Torsolo, 2006).** In our study, IgY was produced from local house breeds against, *E. coli* O157:H7, *S. typhymurium*, *G. anatis* and *S. aureus*. The purity and specificity of IgY was monitored by SDS-PAGE analysis and growth inhibition plus agar gel immunodiffusion for *G. anatis*.

The structure of IgY is significantly different from that of mammalian IgG despite their similarity in function **(Leslie and Clem, 1989; Carlander et al., 1999**). IgY contains two heavy and two light chains and has a molecular mass of 180 kDa, larger than that of mammalian IgG (159 kDa). IgY possesses a larger molecular weight heavy chain (68 kDa) as compared to that from mammals (50 kDa) and two light chains with the molecular mass of 25 KDa each **(Warr et al., 1995).** The purified IgY **(Figure 1)**, showed two major bands with a molecular weight of 26~ 27 KDa and 63~ 69 KDa corresponding to light and heavy chains, respectively. This finding agrees with those reported by **Zhen et al., (2008); Guimaraes et al., (2009); Li et al., (2014); Abo-Ghanema et al., (2016); Nahla et al., (2018)**.

Unlike a study conducted by **Itoh et al., (1986)** who showed that IgY consisted of 74 KDa heavy chain and 28 KDa light chain in SDS-PAGE analysis under reducing conditions. Also, **Bizhanov and Vyhniauskis, (2000)** showed that SDS-PAGE analysis of purified IgY gave three major protein components with molecular weights of 34 KDa, 41KDa and 66 KDa with one minor protein of 45 KDa. In addition, **Nasiri et al., (2016)** showed that IgY contained two major proteins; 23 kDa (light chain) and 68 kDa (heavy chain).

Agar gel immunodiffusion test for *G. anatis* **(Figure 3)** showed precipitation lines that first appeared by the third week with the serum IgY. In contrast, no precipitation lines could be detected between the *G. anatis* antigen and purified yolk IgY at different weeks post immunization. It was clearly shown that immunodiffusion technique has low sensitivity as reported by **Socket et al., (1992); Jithendran et al., (1996); Ferreira et al., (2002)** where, this technique requires high concentration of both antigen and antibodies with low affinities with subsequent inability for earlier detection of antibodies. Thus, as a possible result of this low sensitivity, detection of antibodies in the yolk samples began one week later than in the serum and ceased one week earlier than in serum despite the continuous detection of such antibodies in yolk samples by growth inhibition test throughout the experiment.

Appearance of precipitation line in serum without detection of such lines in yolk samples from the same group may be attributed to the antigen construction of *G. anatis* that belongs to thymus independent antigens which stimulate chickens to produce serum IgM and few serum IgG with subsequent low level of IgY in yolk **(Hung et al., 1987)**.

The inhibition of bacterial growth by serum IgY started from the 1st week after immunization then increased gradually till reaching peak at the 4th week and continued up to the 8th week post immunization. For yolk antibodies the inhibition started from the 2nd week, and increased gradually till reaching peak at 5th week and continued up to the 8th week post immunization. Also, serum antibodies appeared earlier than those of yolk as reported by previous studies (**Abo-Ghanema et al., 2016; Jensinus and Koch., 1997; Ling et al., 1998)** and this finding enables monitoring the level of yolk IgY by testing blood samples taken from immunized hens **(Kritratanasak et al., 2004).**

Zhang et al. (2019) indicated that specific IgY antibody against GtxA-N can be produced and inhibiting *G. anatis* growth efficiently in vitro when compared to antibiotics. Their results indicated that the use of GtxA-N-specific chicken egg yolk antibody may be a valuable approach that could be commercially applied to reduce *G. anatis* infection. Authors indicated that their findings showed that GtxA-N-specific IgY administration may reduce the clinical use of antibiotics and lower the risk of G. anatis developing antibiotic resistance, which in part confirm our results as well.

Maternal antibodies transfer can be defined as the passage of antibodies by mother to her offspring through colostrum milk or egg **(Grindstaff et al., 2003**). Birds transmit maternal antibodies to their offspring by depositing antibodies in their eggs **(Brambell, 1970)**. **Losch et al., (1986); Larsson et al., (1993**) reported that laying hens transfer all serum antibody isotypes including IgG, IgM and IgA to their eggs. Further, two possible routes of transfer exist, one where antibodies in hen's serum are secreted into the maturing egg follicles and thus into the yolk. In the other route, antibodies in the oviduct are incorporated into the egg white along with the secreted albumin. They also reported that IgG transfer to ovarian follicles is receptor dependent and the ovarian receptors allow selective transport of all IgG subpopulations presented by the maternal blood but no IgM or IgA.

The variation in percentage of transfer throughout the experiment may be attributed to several factors as reported by **Coakley et al., (2014)** who revealed that eggs are produced on a 24- hour cycle so as one egg is laid, the yolk for the next day's egg has just been formed. The yolk is thereafter packaged and awaits fertilization

in the reproductive tract. The egg for the day after is still sequestering yolk constituents in the ovary. Therefore, this sequential pattern of egg production over a series of days explains why eggs vary in their antibody levels over the course of the immune response.

The wide range of bacterial affections in poultry, animals as well as human, with the increase of their antimicrobial resistance would greatly necessitates finding alternatives that can be of therapeutic as well as preventive efficiencies. Controlling antimicrobial resistance can help greatly in reducing the zoonotic threat to human by such pathogens. Thus, the present study recommends immunization of hens with *E. coli* O157: H7, *S. typhymurium*, *S. aureus* and *G. anatis* for production of hyperimmune chicken eggs with significant titers of antibodies against such pathogens. More studies are to be done further in order to evaluate the therapeutic, preventive and diagnostic properties of the produced IgYs.

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